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(57) Abstract

The present invention describes methods for site-directed integration of a transgene in a plant genome using site-specific recombination and site-specific recombinase systems. The invention discloses the use of two different recombinase recognition sites, in particular two recombinase recognition sites which differ in their spacers. The invention also relates to transgenic plants obtained by a method of the present invention and to their progeny.

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SITE-DIRECTED TRANSFORMATION OF PLANTS

The invention relates to methods for plant transformation, in particular for the precise integration of foreign DNA into defined sites in the genome of a plant. The invention also relates to transgenic plants obtained by such methods.

In recent years, the development of genetic engineering techniques has had dramatic implications in the field of crop improvement. Using these techniques, beneficial traits can be introduced into almost any crop and such improved crops can be rapidly obtained instead of having to go through the lengthy process of introducing the desired trait by conventional breeding methods.

However, present plant transformation methods generally lead to integration of the transgene at random locations in the host genome. This results in several problems, such as variability of transgene expression caused by different integration loci or so-called "positions effects", and the risk of creating a mutation in the genome upon integration of the transgene into the host genome. Therefore, a large number of transformation events must be screened and tested in order to obtain a transgenic plant exhibiting the desired level of expression of the transgene without producing any abnormality resulting from the insertion of the transgene into an important locus in the host genome. Another disadvantage of random transgene integration is that if additional transgenes are to be subsequently added to a transgenic plant, integration of the additional trait at a genomic location different from the pre-existing transgene renders the breeding of both transgenes in elite lines cumbersome and difficult.

One approach to obtain integration of a transgene at a predetermined location in the host genome is the use of homologous recombination. In this case, the incoming plasmid DNA molecule contains a stretch of DNA sequence identical to a particular genomic sequence and integration of the incoming DNA occurs through reciprocal recognition of the homologous sequences by the normal recombination enzymes of the plant. Although homologous recombination is an efficient mechanism in yeast, fungi and certain types of mammalian cells, frequencies of homologous integration of a transgene in plants are extremely low compared to the background of random integrations, thus making the use of this approach for the genetic transformation of plants not generally feasible.

Therefore, there is an unfulfilled need for improved transformation methods allowing for site-directed integration of a transgene at a predetermined genomic location into the host genome.

The present invention relates to plant transformation and, because of the lack of predictability for the site of integration of a transgene in a plant genome using current transformation methods, the present invention addresses the need for precise modifications of plant genomes. In particular, the present invention provides methods for the integration of transformed DNA into a defined location in the host genome using a site-specific recombinase system and further provides transgenic plants obtained using the methods of the present invention.

Site-specific recombinase systems typically comprise one or more proteins which mediate the specific cleavage and ligation of two defined, asymmetric nucleotide sequences, the recombinase recognition sites. The recombinase recognition sites usually comprise two inverted repeats of about 13-bp separated by a spacer of about 8-bp. In some cases, the recognition site comprises three repeats, arranged as two in direct orientation, the 8-bp spacer and a third repeat in inverted orientation relative to the other two repeats. Because the recombinase mediates recognition and recombination between two recognition sites, if two identical recognition sites are present in a host genome in the continued presence of the corresponding recombinase activity, they can further recombine, thus creating an unstable chromosomal locus which is not desirable. It is therefore an object of the present invention to use combinations of different recombinase recognition sites for site-specific integration of a transgene into the host genome, resulting in a stable chromosomal locus after recombinase-mediated integration of a transgene.

Using a method of the present invention, it is therefore possible to avoid the variability of transgene expression usually caused by "position effects" of transgene insertion in transgenic plants or by different numbers of copies of the transgene integrated into the host genome. Additionally, by using the method of the present invention, the possibility of mutagenesis due to random insertion of the transgene is avoided. Furthermore, the present methods also allow for additional transgenes to be inserted at the predetermined genome location instead of being scattered throughout the genome, thereby, e.g., stacking transgenes at the pre-determined genome location. Furthermore, using a method of the present invention transgenes can be exchanged or no longer useful transgenes can be removed.

The present invention therefore provides:

A method for site-specific modification of a plant genome comprising the steps of: a) obtaining a plant cell stably transformed with a first DNA sequence comprising two recombinase recognition sites which differ from one another in their spacer; b) introducing into said plant cell a second DNA sequence comprising the two recombinase recognition sites corresponding to the recombinase recognition sites of the first DNA sequence, in the presence of active recombinase or recombinases; c) identifying a recombinase-mediated integration of the second DNA sequence at the chromosomal location of the first DNA sequence. In a preferred embodiment, a nucleotide sequence is added at the pre-determined genome location. In another preferred embodiment, a nucleotide sequence at the pre-determined genome location is removed and preferably replaced with another nucleotide sequence.

Active recombinase is either provided to said plant cell as active protein, or as a translatable messanger RNA molecule, or transiently expressed, i.e. said plant cell is stably transformed with a DNA sequence comprising the coding region of at least one recombinase expressible in said plant cell.

The two recombinase recognition sites are desirably derived from two different site-specific recombination systems. More desirably, the two recombinase recognition sites are derived from the same site-specific recombination system. Preferably, the site-specific recombination system is selected from a group consisting of R/*RS*, Gin/*gix* and FLP/*FRT*. More preferably, the site-specific recombination system is FLP/*FRT*. Yet more preferably, the wild-type nucleotide sequence of the spacer is 5'-TCT AGA AA-3' (SEQ ID NO:1) and a modified spacer of a recognition site of the present invention is selected from the group consisting of 5'-TTC AAA AG-3' (SEQ ID NO:2), 5'-TTC AAA TA-3' (SEQ ID NO:3), 5'-TCT ACT TA-3'(SEQ ID NO:4), 5'-TCT AGA AG-3'(SEQ ID NO:5) and 5'-TCT AGA TA-3'(SEQ ID NO:6).

In a preferred embodiment, the first DNA sequence further comprises outside of the DNA stretch comprised between the two recombinase recognition sites the coding region of a selectable maker gene or a portion thereof, preferably preferably a 3' portion of the coding region of the selectable marker gene, wherein the 5' end of said coding region is adjacent to one of the recombinase recognition sites, wherein said selectable marker gene or portion thereof is potentially expressible in said plant cell if a promoter were provided, but not

expressed. In a further preferred embodiment, the 3' end of said coding region is operably linked to termination signals. In a further preferred embodiment, an intron or portion thereof is operably linked to the 5' end of the coding region of a selectable maker gene or portion thereof, wherein the 5' end of said intron of portion thereof is adjacent to one of the recombinase recognition sites. In a further preferred embodiment, the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell. For example the first DNA sequence further comprises an expression cassette comprising a visible or a negative selectable marker gene expressible in said plant cell. In another preferred embodiment, the second DNA sequence further comprises between the two recombinase recognition sites a promoter capable of directing the expression of a gene in a plant cell, wherein said promoter is adjacent to one of the recognition sites and is oriented such that it is capable of directing transcription towards the outside of the DNA stretch comprised between the two recombinase recognition sites, wherein a functional fusion between said coding region of the first DNA sequence and said promoter is created upon recombinase-mediated integration of the second DNA sequence at the chromosomal location of the first DNA sequence. In a further preferred embodiment, the second DNA sequence further comprises between the two recombinase recognition sites a promoter capable of directing the expression of a gene in a plant cell operably linked to a portion of the coding sequence of a selectable marker gene, preferably a 5' portion of the coding region of the selectable marker gene, preferably a portion of the coding region of the selectable marker gene not present in the first DNA sequence, wherein said portion of the coding sequence of the gene of interest fused to said promoter is adjacent to one of the recognition sites and is oriented such that the promoter is capable of directing transcription towards the outside of the DNA stretch comprised between the two recombinase recognition sites, wherein a functional fusion between the portion of the coding region of the selectable marker gene of the first DNA sequence and the portion of the coding region of the selectable marker gene of the second DNA sequence is created upon recombinase-mediated integration of the second DNA sequence at the chromosomal location of the first DNA sequence. In a further preferred embodiment, an intron or portion thereof is operably linked to the 3' end of the portion of the coding region of the selectable maker gene.

In another further preferred embodiment, the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell. In another further embodiment, the second DNA

sequence further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell and differing from the marker in the first DNA sequence. In another further embodiment, the second DNA sequence further comprises between the two recombinase recognition sites one or more additional recombinase recognition site different from the other two recombinase recognition sites in said DNA sequence and adjacent to the 5' end of the coding region of a selectable marker gene. In a preferred embodiment, termination signals are fused to the 3' end of the coding region of the selectable marker gene.

In another preferred embodiment, the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a selectable marker gene expressible in said plant cell. In a further preferred embodiment, the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell, and desirably further comprises an expression cassette comprising a visible or negative selectable marker gene expressible in said plant cell.

In a further preferred embodiment, the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a selectable marker gene expressible in said plant cell. In another further preferred embodiment, the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell. In another further preferred embodiment, the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell. the second DNA sequence further comprises between the two recombinase recognition sites one or more additional recombinase recognition site different from the two other recombinase recognition sites of said DNA sequence.

The method optionally further comprises the step d) of regenerating from a plant cell comprising a DNA sequence as discussed above to obtain a fertile plant.

The invention further provides:

A plant cell stably transformed with a DNA sequence comprising at least two different recombinase recognition sites. In preferred embodiment, the plant cell further comprises outside of two recombinase recognition sites the coding region of a selectable maker gene. wherein the 5' end of said coding region is adjacent to one of the recombinase recognition sites, wherein said selectable marker gene is potentially expressible but not expressed in

said plant cell, and preferably where the 3' end of said coding region is operably linked to termination signals. In a further embodiment, the plant cell further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell.
In another preferred embodiment, the plant cell further comprises between two recombinase recognition sites an expression cassette comprising a selectable marker gene expressible in said plant cell. In a further embodiment, the plant cell further comprises between the two recombinase recognition sites an expression cassette comprising a visible or negative selectable marker gene expressible in said plant cell.

The invention further provides:
A plant comprising plant cells as described above and regenerated from any one of the plant cells described above or a descendant of such a plant having stably integrated in its genome a DNA sequence as described above.

The invention further provides:
A plant obtained by any one of the methods described above or a descendant of such a plant having stably integrated in its genome a DNA sequence as described above.

Within the context of the present invention "site-specific" means at a particular DNA sequence, which is in a specific location in the genome. The nucleotide sequence can be an endogenous nucleotide sequence of the genome at its natural location in the genome or it can be a heterologous nucleotide sequence which has been previously inserted into the genome by any of a variety of known methods.

To "identify" a recombinase-mediated integration means that either a selectable marker gene is used that selects for the site-specific integration or that a visible or negative selectable marker gene is used, whose loss identifies a site-specific integration.

"Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of

the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA that, in the sense or antisense direction, inhibits expression of a particular gene, e.g., antisense RNA. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular DNA sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue or organ or stage of development.
"Heterologous" as used herein means "of different natural or of synthetic origin", i.e. it represents a non-natural state. For example, if a host cell is transformed with a gene derived from another organism, particularly from another species, that gene is heterologous with respect to that host cell and also with respect to descendants of the host cell which carry that gene. Similarly, heterologous refers to a nucleotide sequence derived from and inserted into the same natural, original cell type, but which is present in a non-natural state, e.g. a different copy number, or under the control of different regulatory elements.
"Regulatory elements" refer to sequences involved in conferring the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

A "selectable marker gene" refers to a gene whose expression in a plant cell gives the cell a selective advantage. The selective advantage possessed by the cells transformed with the selectable marker gene may be due to their ability to grow in presence of a negative selective agent, such as an antibiotic or a herbicide, compared to the ability to grow of non-transformed cells. The selective advantage possessed by the transformed cells may also be due to their enhanced capacity, relative to non-transformed cells, to utilize an added compound as a nutrient, growth factor or energy source. A selective advantage possessed

by a transformed cell may also be due to the loss of a previously possessed gene in what is called "negative selection". In this, a compound is added that is toxic only to cells that did not lose a specific gene (a negative selectable marker gene) present in the parent cell (typically a transgene).

A "visible marker gene" refers to a gene whose expression does not confer an advantage to a transformed cell but can be made detectable or visible.

A "gene of interest" refers to any gene which, when transferred to a plant, confers upon the plant a desired characteristic, such as, but not limited to, virus resistance, insect resistance, disease resistance, or resistance to other pests, herbicide tolerance, improved nutritional value, improved performance in an industrial process or altered reproductive capability. The "gene of interest" may also be one that is transferred to plants for the production of commercially valuable enzymes or metabolites in the plant.

A "plant" refers to any plant or part of a plant at any stage of development. Therein are also included cuttings, cell or tissue cultures and seeds. As used in conjunction with the present invention, the term "plant tissue" includes, but is not limited to, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell cultures, and any groups of plant cells organized into structural and/or functional units.

A plant "regenerated" from a plant cell means that all cells of the plant are derived from that plant cell.

I. Site-specific Recombinase Recognition Systems

The present invention relates to methods for targeting the insertion of a transgene into a predetermined chromosomal location using site-directed recombinase systems. A site-specific recombinase system typically comprises one or more proteins which recognize two copies of a specific nucleotide sequence, and cleave and ligate them, thereby providing a precise, site-specific exchange of genetic information. Several site-specific recombinases are known in the art. These include, but are not limited to, e.g., the bacteriophage P1 Cre/*lox* system (Austin et al. (1981) Cell 25: 729-736), the R/*RS* recombinase system from

the pSR1 plasmid of the yeast *Zygosaccharomyces rouxii* (Araki et al. (1985) J. Mol. Biol. 182: 191-203), the Gin/*gix* system of phage Mu (Maeser and Kahlmann (1991) Mol. Gen.Genet. 230: 170-176) and the FLP/*FRT* recombinase system from the 2µm plasmid of the yeast *Saccharomyces cerevisiae* (Broach et al. (1982) Cell 29: 227-234).

Recombinase recognition sites usually comprise two 13-bp inverted repeats separated by an 8-bp spacer. In some cases, the recognition site comprises three repeats, with two repeats in direct orientation, the 8-bp spacer and a third repeat in inverted orientation relative to the other two repeats. The six internal base pairs of the spacer, which is the site of cross-over, are usually not contacted by the recombinase (see e.g. Bruckner and Cox (1986) J. Biol. Chem. 261: 11798-11807) but are important determinants for the specificity of interactions between different spacers within a particular recombinase system (see e.g. Umlauf and Cox (1988) EMBO J. 7: 1845-1852).

Typically, when two recombinase recognition sites are in direct repeat orientation recombinase-mediated recombination results in the deletion of the genetic material located between the two repeats, while recombination between two recognition sites in indirect repeat orientation results in the inversion of the genetic material located between the two recognition sites. Additionally, interactions between recombinase recognition sites located on two different molecules is possible and since some of these recombinase systems have been shown to function properly in heterologous hosts, such as mammals and plants, they potentially represent a powerful tool for precise, site-specific modification of plant genomes.

II. Use of Different Recombinase Recognition Sites

If two identical recognition sites are present in the host genome after a recombinase-mediated recombinase event and if an active recombinase is still available in the host cells, the two recognition sites are likely to continue to recombine, thereby creating an unstable locus. Furthermore, since intramolecular excision is kinetically favored over bi-molecular integration (Albert et al. (1995) Plant J. 7: 649-659), proper integration events can be lost before the plant cells become free of recombinase.

A strategy to avoid reversion at the modified chromosomal locus is to provide the recombinase only transiently (see e.g. Fukushige and Sauer (1992) PNAS 89: 7905-7909) but, due to the unfavored kinetic of integration vs. excision, this strategy has only limited potential. Another strategy consists of creating mutant recognition sites which kinetically favor insertion vs. excision, such as *lox* sites containing mutated inverted repeats (Albert et

al. (1995) Plant J. 7: 649-659). In this case, the mutations were restricted to the inverted repeats but only three mutated recognition sites were shown to have improved kinetics regarding insertion vs. excision without loosing their general recombination abilities. As there did not seem to be any predictability in the discovery of the three mutated recognition sites, this strategy may require intensive experimentation in order to be applied to other recombinase systems. Furthermore, recombinase recognition sites modified in their inverted repeats are still able to further recombine.

It is therefore an object of the present invention to use combinations of different recombinase recognition sites, which result in a stable chromosomal locus after recombinase-mediated integration of a transgene. In a preferred embodiment, the two different recombinase recognition sites are derived from two different recombinase systems. The two different recombinase recognition sites differ in their sequences, in particular in their inverted repeats and in their spacers. In this case, the two active recombinases are provided to the recipient cells in order to obtain site-specific recombination. For example, one site is a *RS* site while the other is a *FRT* site and active R and FLP protein are provided, but any combination of recombinase recognition sites and corresponding recombinases from different recombinases is contemplated for use in the present invention. In a further preferred embodiment, the two recognition sites are derived from the same recombination system and, in this case, the two sites differ in their spacers. For example, when Cre/*lox* is used, at least one of the spacers of the *lox* sites is modified, when Gin/*gix* is used, at least one of the spacers of the *gix* sites is modified, when R/*RS* is used, at least one of the spacers of the *RS* sites is modified, when FLP/*FRT* is used, at least one of the spacers of the *FRT* sites is modified. In a further preferred embodiment, the FLP/*FRT* site-specific recombinase system is used and the two recombinase recognition sites are *FRT* sites. In this case, the wild-type nucleotide sequence of the spacer is 5'-TCT AGA AA-3' (SEQ ID NO:1). In a preferred embodiment, a desired modified recognition site should not affect the polypyrimidine and polypurine tracts which extend from the spacer's center into the inverted repeats and reduction of the high AT content should be avoided. Preferably, a modified recognition site of the present invention is 5'-TTC AAA AG-3' (SEQ ID NO:2). Other preferred modified recognition sites are 5'-TTC AAA TA-3' (SEQ ID NO:3), 5'-TCT ACT TA-3' (SEQ ID NO:4), 5'-TCT AGA AG-3' (SEQ ID NO:5) or 5'-TCT AGA TA-3' (SEQ ID NO:6). In a further preferred embodiment, the R/*RS* site-specific recombinase system is used and the two recombinase recognition sites are *RS* sites. In a

further preferred embodiment, a wild-type *RS* site comprises the DNA sequence 5'-ACG TT-3', preferably 5'-CGT-3'.

III. Evaluation of the Recombinase Recognition Sites in Plants

It is known in the art that plants are different from other kingdoms in many aspects and that results obtained in different kingdoms cannot predictably be extrapolated to plants. Therefore, the inventors of the present invention first tested the element of the site-specific recombinase system in plants, in particular the recombinase recognition sites.
The FLP recombinase responsive reporter gene constructs are assayed in the presence and absence of FLP recombinase. A stuffer fragment surrounded by two recombinase recognition sites is inserted between the coding sequence of a luciferase gene and a promoter. The stuffer fragment flanked by *FRT* sites is excised from the untranslated leader of the luciferase gene only in the presence of FLP recombinase, resulting in transcription of the luciferase gene and luciferase activity. For the wild-type *FRT* sites the luciferase activity in presence of active recombinase is 73-fold over the luciferase activity in the absence of the recombinase. The modified F^3 and F^5 sites are also recognized by FLP recombinase, and in the presence of the recombinase the stuffer fragments flanked by F^3 and F^5 sites are also excised from the untranslated leader of the luciferase gene, resulting in luciferase activity of 18-fold and 38-fold respectively over that of pAT206 and pAT207 in the absence of recombinase (compare example 3).
The cross-reactivity between wild-type and modified *FRT* sites is also tested. Luciferase activity in the presence of FLP recombinase is tested on constructs containing stuffer fragments flanked by wild-type and F^5 sites, wild-type and F^3 sites and F^3 and F^5 sites. (pAT210, 211 and 213). Luciferase activity of these constructs in the presence of recombinase is only two-fold over that of the pAT163 vector (stuffer flanked by wild-type sites) in the absence of recombinase, demonstrating that combinations of the wild-type and modified *FRT* sites are not cross-reactive (compare example 3).
For the first time, the inventors of the present invention show that these elements are functional and interact specifically in plants.

IV. Recipient Lines and Donor Constructs

The present invention provides a method to carry out site-specific integration of a transgene using two different recombination recognition sites and comprising the steps of: a) obtaining a plant cell (recipient cell) stably transformed with a first DNA sequence (recipient construct) comprising two recombinase recognition sites which differ from one another in their spacer; b) introducing into said plant cell a second DNA sequence (donor construct) comprising the two recombinase recognition sites corresponding to the recombinase recognition sites of the first DNA sequence, in the presence of active recombinase; c) selecting and screening for a recombinase-mediated integration of the second DNA sequence at the chromosomal location of the first DNA sequence.

A recipient cell of the present invention is obtained by a transformation method as described below or by methods otherwise known in the art. Such transgenic line preferably contains a single copy of the recipient construct integrated in its genome. Once such a line has been identified, it is further characterized. For example, the location of the transgene insertion is precisely determined by genetic methods well known in the art. Additionally, the host plant DNA flanking the site of insertion is sequenced and it is ascertained that no essential gene is mutated by the insertion of the transgene. Once a well characterized line is obtained, it is used as a recipient for subsequent introduction of additional transgenes. Such additional transgenes are comprised in the donor construct and are introduced into the recipient line by transformation methods as described below or otherwise known in the art.

In a preferred embodiment, the two recombinase recognition sites are in direct orientation in the DNA sequence used to produce the recipient line. In a further preferred embodiment, the coding region of a selectable marker gene and preferably termination signals fused at the 3' end of the coding region are comprised in the DNA sequence precisely situated outside of one of the recombinase target sites flanking the DNA stretch located between the two recombinase recognition sites (Fig. 2). The 5' end of the coding region is adjacent to one of the recombinase recognition sites. In a preferred embodiment, the recombinase recognition site is inserted in an intron located in the untranslated leader at the 5' end of the coding region. In another preferred embodiment, the recombinase recognition site is inserted in the 5' untranslated region of the selectable marker gene. In another preferred embodiment, a portion of the coding region of the selectable marker

gene, preferably a 3' portion, is used, wherein the 5' end of the portion of the coding region of the selectable marker gene is adjacent to one of the recombinase recognition site. In a further preferred embodiment, an intron or portion thereof is operably linked to the 5' end of the coding region of the selectable maker gene or to a portion thereof, wherein the 5' end of said intron or portion thereof is adjacent to one of the recombinase recognition sites. In such construct, no transcription of the selectable marker gene is possible because of the absence of a promoter directing the expression of the selectable marker gene. Such selectable marker gene can be any selectable marker gene described below or otherwise known in the art. In a preferred embodiment, such selectable marker gene is the *bar* gene. In a further preferred embodiment, the DNA sequence also comprises an expression cassette which is included between the two recombinase recognition sites in the DNA sequence. The expression cassette preferably comprises a selectable marker gene, such as any selectable marker gene described below or otherwise known in the art. In a preferred embodiment, the selectable marker gene is an *nptII* gene, conferring resistance to kanamycin and a preferred promoter in the expression cassette is the *nos* promoter. The DNA sequence desirably also further comprises another expression cassette which comprises a gene of interest and desirably also further comprises a visible marker gene, such as, e.g., a GUS gene, a luciferase gene or a GFP gene, or another selectable marker gene. The DNA sequence preferably also further comprises an expression cassette comprising a negative selectable marker gene, such as a cytosine deaminase gene (Perera et al. (1993) PMB 23: 793-799), a Herpes Simplex Virus Thymidine Kinase gene (Czako and Marton (1994) Plant Physiol. 104: 1067-1071), a T-DNA gene 2 (Depicker et al. (1988) Plant Cell Reports 7: 63-66).

The donor construct used to transform the recipient line described above comprises the corresponding two recombinase recognition sites. In a preferred embodiment, the two recombinase recognition sites in such donor construct are in direct repeat orientation. In a further preferred embodiment, the donor construct comprises a promoter located between the two recombinase recognition sites, wherein the promoter is adjacent to one of the recognition sites and is capable of directing transcription towards outside of the DNA stretch comprised between the two recombinase recognition sites. In a further preferred embodiment, the promoter is operably linked to a portion of the coding sequence of a selectable marker gene, preferably a 5' portion of the coding region of the selectable marker gene, preferably a portion of the coding region of the selectable marker gene not present in the recipient construct. In a further preferred embodiment, an intron or portion thereof is

operably linked to the 3' end of the portion of the coding region of the selectable maker gene.

A promoter for such construct is any promoter described below or otherwise known in the art. Preferably, such promoter is the ubiquitin 3 (*UBQ3*) promoter (Norris et al. (1993) PMB 21: 895-906). The donor construct also comprises an expression cassette comprising a selectable marker gene inserted between the two recombinase recognition sites. Such selectable marker gene is any selectable marker gene described below or otherwise known in the art but is different from a selectable marker gene comprised in the recipient line. In a preferred embodiment, such selectable marker gene is the hygromycin resistance gene. The donor construct also desirably comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene, such as, e.g., a GUS gene, a luciferase gene or a GFP gene. Additionally, the donor construct also desirably comprises one or more additional recombinase recognition site, which is different from the other two recombinase recognition sites and which is adjacent to the 5' end of the coding region of a selectable marker gene. Such selectable marker gene is any selectable marker gene described below or otherwise known in the art but is different from a selectable marker gene comprised in the recipient line. In a preferred embodiment, termination signals are fused to the 3' end of the coding region of the selectable marker gene. The third recombinase recognition site and the coding region of the selectable marker gene are located between the first two recognition sites. Preferably, the third recombinase recognition site is surrounded by the two expression cassettes mentioned *supra*. After a first round of recombinase-mediated integration, the third recombinase recognition site allows for recombinase-mediated integration of further additional transgenes using the third recombinase recognition site and either one of the first two recognition sites. The donor construct also further comprises between the two recombinase recognition sites an additional expression cassette comprising any gene of interest which is to be integrated into the genome of the recipient line. In such expression cassette, the expression of the gene of interest is controlled by any one of the promoters described below.

Upon recombinase-mediated integration of the donor construct, the nucleotide sequence comprised between the two recombinase recognition sites in the recipient line is replaced by the nucleotide sequence of the donor construct, which is located between the two recognition sites in the donor construct. The desired integration event is recovered by selecting for resistance to the selectable marker gene. When the coding sequence of the selectable marker gene is located outside of the DNA stretch comprised between the two

recombinase recognition sites in the recipient line, a functional transcription unit is created between the coding region of the selectable marker gene and the promoter of the donor construct located between the two recombinase recognition sites. When portions of the coding region of the selectable marker gene are used, a functional selectable marker gene is created upon recombinase-mediated integration of the donor construct. When an intron or portions thereof are included in the donor and recipient constructs, a functional selectable marker gene comprising a spliceable intron is created upon recombinase-mediated integration of the donor construct.

In addition to selection for resistance to the selectable marker gene located outside of the DNA stretch comprised between the two recombinase recognition sites in the recipient line, or as an alternative, site-specific recombinase-mediated integration is monitored by the loss of the negative selectable marker gene located between the recombinase recognition sites in the recipient line.

In another embodiment, a recipient line transformed with a different DNA sequence is obtained. Preferably, the two different recombinase recognition sites are in direct orientation in the DNA sequence used to produce the recipient line. In a further embodiment, such DNA sequence also comprises an expression cassette which is included between the two recombinase recognition sites in the DNA sequence. The expression cassette comprises a selectable marker gene, such as any selectable marker gene described below or otherwise known in the art. In a preferred embodiment, the selectable marker gene is a kanamycin resistance gene and a preferred promoter in the expression cassette is the *nos* promoter. The DNA sequence desirably also comprises another expression cassette between the two recombinase recognition sites, which comprises a gene of interest, and also desirably further comprises a visible marker gene, such as, e.g., a GUS gene, a luciferase gene or a GFP gene, or another selectable marker gene. The DNA sequence preferably also further comprises an expression cassette comprising a negative selectable marker gene. The DNA sequence is transformed into a suitable host plant cell and a selectable marker of the DNA sequence is used to select tranformation events.

A donor construct used to transform a recipient line as described immediately above comprises the corresponding two different recombinase recognition sites. In a preferred embodiment, the two recombinase recognition sites in such donor construct are in direct orientation. The donor construct also comprises an expression cassette comprising a selectable marker gene inserted between the two recombinase recognition sites. Such a

selectable marker genes is any selectable marker gene described above or otherwise known in the art, but is different from the selectable marker gene comprised in the recipient line. The donor construct also desirably comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene, such as, e.g., a GUS gene, a luciferase gene or a GFP gene. Additionally, the donor construct also desirably comprises a third recombinase recognition site, which is different from the other two recombinase recognition sites, between the first two recognition sites. Preferably, the third recombinase recognition site is surrounded by the two expression cassettes mentioned *supra*. After a first round of recombinase-mediated integration, the third recombinase recognition site allows for recombinase-mediated integration of further additional transgenes using the third recombinase recognition site and any one of the first two recognition sites. The donor construct also further comprises between the first two recombinase recognition sites an additional expression cassette comprising any gene of interest which is to be integrated into the genome of the recipient line. In such an expression cassette, the expression of the gene of interest is controlled by any one of the promoters described below. Such gene of interest is an insect resistance gene, a disease resistance gene, a herbicide resistance gene or a gene encoding a value-added trait.

Upon recombinase-mediated integration of the donor construct, the nucleotide sequence located between the two recombinase recognition sites in the recipient line is replaced by the nucleotide sequence of the donor construct which is located between the two recognition sites in the donor construct. The occurence of the desired integration event is monitored by resistance of a host cell to a selectable marker gene of the donor construct or lack of resistance to a selectable marker gene or lack of expression of a visible marker gene previously integrated in the recipient line, and which was lost due to the site-specific integration event. In addition to selection for resistance to the selectable marker gene located between the two recombinase recognition sites in the donor construct, or as an alternative, site-specific recombinase-mediated integration is monitored by the loss of the negative selectable marker gene which is located between the recombinase recognition sites in the recipient line.

V. Supply of Active Recombinase

An active recombinase is provided to cells of the recipient line to catalyze the site-specific recombination. In a preferred embodiment, a DNA sequence comprising the coding

sequence of the recombinase is used to deliver the recombinase. In this case, the coding region of the recombinase is comprised in an expression cassette, such that the recombinase is expressible in a host cell of the recipient line and such that an active recombinase is produced. Preferably, the coding region of the recombinase is expressible in a host cell in which the recombinase-mediated integration is taking place. In a further preferred embodiment, any one of the promoters described below is used to direct expression of the recombinase in the desired host cell. In a preferred embodiment, the DNA molecule encoding the recombinase is co-transformed with the donor construct by a method known in the art or described below. Different amounts of recombinase and various molar ratios of donor construct vs. recombinase are used. Desirably, a 1:1 molar ratio is used. A 1:5, 1:10 or 1:100 molar ratio donor construct vs. recombinase is also desirably used if more active recombinase is necessary. A 5:1, 10:1 or 100:1 molar ratio donor construct vs. recombinase is also desirably used if more donor construct is necessary.
In another preferred embodiment, the recipient line is stably transformed with a DNA sequence comprising an expression cassette comprising the coding sequence of a recombinase, wherein such coding sequence is expressible in cells of such recipient line and express an active recombinase. In a preferred embodiment, a constitutive promoter is used. In another preferred embodiment, an inducible promoter is used, such as, e.g., a heat-shock promoter. In yet another preferred embodiment, an excisable expression cassette comprising the coding sequence of a recombinase is used. In a preferred embodiment, in such an expression cassette the coding sequence of a recombinase is flanked by parallel matching sites, for example parallel matching FRT sites. Upon expression of the recombinase site-specific integration of a gene of interest is achieved and excision of the recombinase expression cassette from the host genome is also obtained.

In another preferred embodiment, the recombinase is provided transiently to the plant cells. In a preferred embodiment, the coding sequence of the recombinase is comprised in a viral replicon capable of autonomous replication in cells of the recipient line. In this case, the coding sequence of the recombinase is comprised in an expression cassette which allows expression of the recombinase in cells of the recipient line. Viral replicons are for example derived viruses, e.g. from geminiviruses, such as from the Maize streak virus (Shen and Hohn (1995) J Gen Virol 76:965-969) and provided substantially as described in co-pending application 08/717,676, from the Wheat dwarf virus (Matzeit et al. (1991) Plant Cell 3:247-258), from a tobacco gemini virus such as, e.g. the tobacco golden mosaic virus, or from

other viruses. In a preferred embodiment, the viral replicon comprising the recombinase coding region is introduced into cells of the recipient line by Agroinfection (Grimsley et al. (1989) Mol Gen Genet 217:309-316).

In a further preferred embodiment, the coding sequence of the recombinase is supplied to the recipient cells as a messenger RNA (mRNA). The advantage of such technique is that the recombinase is only provided to the host cells transiently. The coding sequence of the recombinase is inserted in a vector for *in-vitro* transcription of the RNA using methods described in Lebel et al. (1995) Theor. Appl. Genet. 91: 899-906 or in co-pending application 08/717,676. The RNA is then transformed into cells of the recipient line, preferably by co-transformation with the donor construct. In a preferred embodiment, the RNA is transferred to the host cells using particle bombardment as described in co-pending application 08/717,676. In another preferred embodiment, the RNA is introduced into the protoplasts or other cells of the recipient line by PEG-mediated transformation as described in Lebel et al. (1995) Theor. Appl. Genet. 91: 899-906 or by electroporation. In another preferred embodiment, other techniques, such as microinjection of the RNA, are used.

In another preferred embodiment, the active recombinase is supplied to the cells as a purifed protein which is introduced into the cells of the recipient line, for example by microinjection, preferably together with the donor construct (see e.g. Neuhaus et al. (1993) Cell 73:937-952).

A method of the present invention also provides for improved transformation efficiency due to more efficient interactions between the recombinase recognition sites in presence of active recombinase. For example, improved transformation efficiency is achieved for meristem transformation, thereby avoiding the need to go through tissue culture in some crops.

VI. Selectable Marker Genes

Several selectable marker genes are available for the present invention but are not limited to those described below. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene which confers resistance to kanamycin and related antibiotics (Messing & Vierra, Gene 19: 259-268 (1982); Bevan *et al.*, Nature 304:184-187 (1983)), the *bar* gene which confers resistance to the herbicide phosphinothricin (White *et al.*, Nucl Acids Res 18: 1062 (1990), Spencer *et al.* Theor Appl Genet 79: 625-631(1990)), the *hph*

gene which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), and the *dhfr* gene, which confers resistance to methotrexate (Bourouis *et al.*, EMBO J. 2: 1099-1104 (1983). Selection markers resulting in positive selection, such as a phosphomannose isomerase gene, and described in patent application 08/527,474, are also used.

VII. Plant Transformation

Plants transformed in accordance with the present invention may be monocots or dicots and include, but are not limited to, maize, wheat, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugarbeet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, *Arabidopsis thaliana*, and woody plants such as coniferous and deciduous trees, especially maize, wheat, or sugarbeet.

Once a desired DNA sequence has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques.

For their expression in transgenic plants, a DNA sequence may require modification and optimization. It is known in the art that all organisms have specific preferences for codon usage, and the codons in the nucleotide sequence comprised in the DNA molecules of the present invention can be changed to conform with specific plant preferences, while maintaining the amino acids encoded thereby. Furthermore, high expression in plants is best achieved from coding sequences which have at least 35% GC content, and preferably more than 45%. Nucleotide sequences which have low GC contents may express poorly due to the existence of ATTTA motifs which may destabilize messages, and AATAAA motifs which may cause inappropriate polyadenylation. Although preferred gene sequences may be adequately expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al.* Nucl. Acids Res. 17: 477-498 (1989)). In addition, the nucleotide sequences are screened for the existence of illegitimate splice sites which cause message

truncation. All changes required to be made within the nucleotide sequences such as those described above are made using well known techniques of site directed mutagenesis, PCR, and synthetic gene construction using the methods described in the published patent applications EP 0 385 962, EP 0 359 472, and WO 93/07278. If necessary, these modifications are performed on a nucleotide sequence of the present invention, in particular the coding region of a recombinase, of a marker gene or of any gene of interest to be used in a plant of the present invention.

For efficient initiation of translation, sequences adjacent to the initiating methionine may require modification. For example, they can be modified by the inclusion of sequences known to be effective in plants. Joshi has suggested an appropriate consensus for plants (NAR 15: 6643-6653 (1987)) and Clontech suggests a further consensus translation initiator (1993/1994 catalog, page 210). These consensuses are suitable for use with the nucleotide sequences of this invention. The sequences are incorporated into constructions comprising the nucleotide sequence, up to and including the ATG (whilst leaving the second amino acid unmodified), or alternatively up to and including the GTC subsequent to the ATG (with the possibility of modifying the second amino acid of the transgene). If necessary, these modifications are performed on a nucleotide sequence of the present invention, in particular the coding region of a recombinase, of a marker gene or of any gene of interest to be used in a plant of the present invention.

The DNA molecules in transgenic plants are driven by a promoter shown to be functional in plants. The choice of promoter will vary depending on the temporal and spatial requirements for expression, and also depending on the target species. Although many promoters from dicotyledons have been shown to be operational in monocotyledons and *vice versa*, ideally dicotyledonous promoters are selected for expression in dicotyledons, and monocotyledonous promoters for expression in monocotyledons. However, there is no restriction to the provenance of selected promoters; it is sufficient that they are operational in driving the expression of the DNA molecules in the desired cell.

Preferred promoters which are expressed constitutively include promoters derived from Agrobacterium opine synthase genes, e.g. the nos promoter, or a dual promoter from the Agrobacterium Ti plasmid (Velten et al. (1984) EMBO J. 3: 2723-2730), or viral promoters operable in plants, e.g. the CaMV 35S and 19S promoters, and promoters from genes encoding actin or ubiquitin. Another prefered promoter is a synthetic promoter, such as the Gelvin Super MAS promoter (Ni et al. (1995) Plant J. 7: 661-676). The DNA molecules of this invention can also be expressed under the regulation of promoters which are chemically

regulated. Preferred technology for chemical induction of gene expression is detailed in the published application EP 0 332 104 and US patent 5,614,395. A preferred promoter for chemical induction is the tobacco PR-1a promoter.

A preferred category of promoters is that which is wound inducible. Numerous promoters have been described which are expressed at wound sites and also at the sites of phytopathogen infection. Preferred promoters of this kind include those described by Stanford *et al.* Mol. Gen. Genet. 215: 200-208 (1989), Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), and Warner *et al.* Plant J. 3: 191-201 (1993).

Preferred tissue specific expression patterns include green tissue specific, root specific, stem specific, and flower specific. Promoters suitable for expression in green tissue include many which regulate genes involved in photosynthesis and many of these have been cloned from both monocotyledons and dicotyledons. A preferred promoter is the maize PEPC promoter from the phosphoenol carboxylase gene (Hudspeth & Grula, Plant Molec. Biol. 12: 579-589 (1989)). A preferred promoter for root specific expression is that described by de Framond (FEBS 290: 103-106 (1991); EP 0 452 269) and a further preferred root-specific promoter is that from the T-1 gene provided by this invention. A preferred stem specific promoter is that described in US patent 5,625,136 and which drives expression of the maize *trpA* gene, now know as maize indole synthase (see Frey et al. (1997) Science 277: 696- ; Melanson et al. (1997) PNAS 94: 13345).

Preferred embodiments of the invention are transgenic plants expressing a DNA molecule in a root-specific fashion. Further preferred embodiments are transgenic plants expressing the DNA sequence in a wound-inducible or pathogen infection-inducible manner.

In addition to the selection of a suitable promoter, constructions for expression of the coding regions of the present invention in plants preferably require an appropriate transcription terminator to be attached downstream of the heterologous nucleotide sequence. Several such terminators are available and known in the art (*e.g. tm1* from CaMV, E9 from *rbcS*) Any available terminator known to function in plants can be used in the context of this invention.

Numerous other sequences can be incorporated into expression cassettes for the expression of a coding region of this invention. These include sequences which have been

shown to enhance expression such as intron sequences (*e.g.* from *Adh1* and *bronze1*) and viral leader sequences (*e.g.* from TMV, MCMV and AMV).

It may be preferable to target expression of a DNA sequence to different cellular localizations in the plant. In some cases, localization in the cytosol may be desirable, whereas in other cases, localization in some subcellular organelle may be prefered. Subcellular localization of transgene encoded enzymes can be undertaken using techniques well known in the art. Typically, the DNA encoding the target peptide from a known organelle-targeted gene product is manipulated and fused upstream of the nucleotide sequence. Many such target sequences are known for the chloroplast and their functioning in heterologous constructions has been shown.

Vectors suitable for plant transformation are described elsewhere in this specification. For *Agrobacterium*-mediated transformation, binary vectors or vectors carrying at least one T-DNA border sequence are suitable, whereas for direct gene transfer any vector is suitable and linear DNA containing only the construction of interest may be preferred. In the case of direct gene transfer, transformation with a single DNA species or co-transformation can be used (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

The recombinant DNA sequences can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway et al., BioTechniques 4:320-334 (1986)), electroporation (Riggs et al, Proc. Natl. Acad. Sci. USA 83:5602-5606 (1986), *Agrobacterium*-mediated transformation (Hinchee et al., Biotechnology 6:915-921 (1988)), direct gene transfer (Paszkowski et al., EMBO J. 3:2717-2722 (1984)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (see, for example, Sanford et al., U.S. Patent 4,945,050; and McCabe et al., Biotechnology 6:923-926 (1988)). Also see, Weissinger et al., Annual Rev. Genet. 22:421-477 (1988); Sanford et al., Particulate Science and Technology 5:27-37 91987)(onion); Christou et al., Plant Physiol. 87:671-674 (1988)(soybean); McCabe et al., Bio/Technology 6:923-926 (1988)(soybean); Datta et al., Bio/Technology 8:736-740 (1990)(rice); Klein et al., Proc. Natl. Acad. Sci. USA, 85:4305-4309 (1988)(maize); Klein et al., Bio/Technology 6:559-563 (1988)(maize); Klein et al., Plant Physiol. 91:440-444 (1988)(maize); Fromm et al., Bio/Technology 8:833-839 (1990); and Gordon-Kamm et al., Plant Cell 2:603-618 (1990)(maize); Svab et al. Proc. Natl. Acad. Sci. USA 87: 8526-8530 (1990) (tobacco chloroplast); Koziel *et al.* (Biotechnology 11:

194-200 (1993)) (maize); Shimamoto *et al.* Nature 338: 274-277 (1989) (rice); Christou *et al.* Biotechnology 9: 957-962 (1991) (rice); European Patent Application EP 0 332 581 (orchardgrass and other *Pooideae*); Vasil *et al.* (Biotechnology 11: 1553-1558 (1993) (wheat); Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993) (wheat). Pollen transformation (US Patent 5,629,183) is also used in the present invention.

One particularly preferred set of embodiments for the introduction of recombinant DNA sequences into maize by microprojectile bombardment can be found in U.S. Serial No. 08/008,374, herein incorporated by reference in its entirety. An additional preferred embodiment is the protoplast transformation method for maize as disclosed in European Patent Application EP 0 292 435, as well as in U.S. Serial Number 08/024,875, hereby incorporated by reference in its entirety.

EXAMPLES

Example 1: **Construction of a FLP Expression Vector for Plants**

The FLP recombinase coding sequence is obtained from pOG44, a mammalian expression vector (Stratagene, LaJolla, CA). A vector containing only the coding sequence is created by ligating the 0.76 kb *HindIII-EcoRI* and the 0.63 kb *EcoRI-KpnI* fragments of pOG44 into the *KpnI-HindIII* sites of pLITMUS 29 (New England Biolabs, Beverly, MA). In order to place a *BglII* site from a polylinker at the 3' end of the gene, a *BamHI-SnaBI* fragment is inserted into the *BamHI-EcoRV* sites of pSGCJH4. An *Arabidopsis thaliana* polyubiquitin gene promoter (*UBQ3*, Norris et al. (1993) PMB 21:895-906) fusion to FLP recombinase is constructed by ligation of the *BamHI-BglII* fragment from the previous vector into the *BamHI* site of pCIB7813 (*UBQ3* promoter and intron in the 5' nontranslated region with a *BamHI* site before the *nos* terminator. This FLP recombinase plant expression vector is pAT152. In order to make a non-functional FLP recombinase as a control for random versus site-directed integration, the *EcoRI* site in the FLP recombinase coding sequence is filled in with Klenow, creating a frame-shift mutation. The mutated recombinase is fused to the *UBQ3* promoter as above and is known as pAT153.

Example 2: **Construction of Vectors for Transient Assays Containing Wild-type and Modified *FRT* Sites**

A vector containing a 1.4kb stuffer fragment (neomycin resistance cassette) flanked by *FRT* sites inserted into the untranslated leader of a luciferase gene is constructed to test the excision efficiency of FLP recombinase on wild-type *FRT* sites in plants. The 35S leader and luciferase gene are excised from pPH121 as a *BamHI-XbaI* fragment and ligated in a three-way ligation to a *SpeI-HindIII nos* terminator fragment and *BamHI-HindIII* cut pbluescript SK+, creating pAT155. A wild-type *FRT* site with *BamHI-BglII* sticky ends is assembled by annealing and kinasing two oligonucleotides: 5'-GAT CCG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCG AAT TCC A-3' (SEQ ID NO:7) and 5'-GAT CTG GAA TTC GAA GTT CCT ATA CTT TCT AGA GAA TAG GAA CTT CGG AAT AGG AAC TTC G-3' (SEQ ID NO:8). The *FRT* site is inserted before the 35S leader by ligation of the *BamHI-BglII* double-stranded oligonucleotide into the *BamHI* site of pAT155 to create pAT161. The Arabidopsis *UBQ3* promoter and leader from pPH121 is inserted as a *BamHI* fragment into the *BamHI* site of pAT161, yielding pAT162. In order to disrupt luciferase expression, a *XbaI* fragment from pNEObGAL (Stratagene, La Jolla, CA) containing a neomycin resistance cassette flanked by *FRT* sites is inserted into the *XbaI* site of pAT162, resulting in pAT163. The *XbaI* sites are in the spacer region of the *FRT* site and insertion of the pNEObGAL *XbaI* fragment into pAT162 recreates a stuffer fragment flanked by single *FRT* sites in the same orientation.

Vectors are also constructed with combinations of wild-type and modified *FRT* sites (F^3 and F^5, Schlake and Bode (1994) Biochemistry 33: 12,746-12,751) flanking a 1.4kb stuffer fragment (neomycin resistance cassette) inserted into the untranslated leader of a luciferase gene in order to test the excisional activity of FLP recombinase on the *FRT* sites with modified spacers and the cross-reactivity between different *FRT* sites in the presence of active recombinase. The following *FRT* sites containing *BamHI-BglII* sticky ends are assembled by annealing and kinasing two oligonucleotides: a F^3 modified *FRT* site: 5'-GAT CCG AAG TTC CTA TTC CGA AGT TCC TAT TCT TCA AAT AGT ATA GGA ACT TCA-3' (SEQ ID NO:9) and 5'-GAT CTG AAG TTC CTA TAC TAT TTG AAG AAT AGG AAC TTC GGA ATA GGA ACT TCG-3' (SEQ ID NO:10), a F^5 modified *FRT* site: 5'-GAT CCG AAG TTC CTA TTC CGA AGT TCC TAT TCT TCA AAA GGT ATA GGA ACT TCA-3' (SEQ ID NO:11) and 5'-GAT CTG AAG TTC CTA TAC CTT TTG AAG AAT

AGG AAC TTC GGA ATA GGA ACT TCG-3' (SEQ ID NO:12) and a wild-type *FRT* site: 5'-GAT CCG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCA-3' (SEQ ID NO:13) and 5'-GAT CTG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCG-3' (SEQ ID NO:14). A *BamHI-XhoI* fragment from pNEObGAL containing the 3' part of the neomycin resistance cassette was subcloned into the respective sites of pLIT28, creating pAT184. The wild-type, F^3 and F^5 *FRT* sites were inserted into the *BamHI* site of pAT184, creating pAT189 (wild-type *FRT* site), pAT187 (F_3 *FRT* site) and pAT186 (F_5 *FRT* site). PCR was performed to add a *BglII* site to the 5' end of the neomycin resistance cassette from pNEObGAL using oligonucleotides 5'-ACA CGG CGG CAT CAG AGC AG-3' (SEQ ID NO:15) and 5'-TAA CAG ATC TCC CCC TGG CGA AAG G-3' (SEQ ID NO:16) and the product is cloned directly into the pGEM-T Easy vector (Promega, Madison, WI), creating pAT190. The *BglII* site of pAT190 is used to insert the *BamHI-BglII* wild-type *FRT* site (pAT199), the F^3 *FRT* site (pAT197) and the F^5 *FRT* site (pAT196). In order to insert the neomycin resistance cassette flanked by *FRT* sites in the untranslated leader of the luciferase gene, a vector with a unique *BamHI* site in the leader is created. pAT175 is constructed by a three-way ligation of an *XbaI-BamHI* fragment of pCIB7812 (Arabidopsis *UBQ3* promoter and leader) and *BamHI-EcoRI* fragment from pAT155 (35S leader, luciferase gene and nos terminator) into *XbaI-EcoRI* cut pUC19. Into this unique *BamHI* site of pAT175 is inserted: a *BamHI-XhoI* fragment from pAT186 and a *XhoI-BglII* fragment from pAT196 to create pAT206 (F^5 *FRT* sites flanking neomycin resistance cassette), the respective fragments from pAT187 and pAT197 into pAT175 to create pAT207 (F^3 *FRT* sites flanking the stuffer cassette), the respective fragments from pAT189 and 196 into pAT175 to create pAT210 (wild-type and F^5 *FRT* sites flanking the stuffer), the respective fragments from pAT189 and 197 into pAT175 to create pAT211 (wild-type and F^3 *FRT* sites flanking the stuffer fragment) and the respective fragments from pAT187 and 196 into pAT175 to create pAT213 (F^3 and F^5 *FRT* sites flanking the stuffer fragment). The *FRT* sites flanking the stuffer fragments in pAT206-213 are in the same orientation.

Example 3: **Tobacco Transient Expression Experiments**

Tobacco transient assays are performed to test the excisional activity of FLP recombinase on the wild-type and modified *FRT* sites and the cross-reactivity between different *FRT* sites. Tobacco leaf mesophyll protoplasts are transformed by the polyethylene glycol-mediated method as described by Shillito and Saul (Plant Molecular Biology: A Practical Approach, C.H. Show eds. IRL Press, Chapter 7, pp. 161-186) with the following modifications. The heat shock treatment and carrier DNA addition steps are omitted. The protoplasts are resuspended at 1 x 10^6 protoplasts/ml, 25 mg of each plasmid is added and PEG solution is added to a final concentration of 16.5%. The protoplast pellets are resuspended in 2.0 mls of K3A medium and incubated in the dark at 24-26 ^{o}C for 24-27 hours. The protoplasts are diluted with 8 mls of W5, centrifuged and the pellets resuspended in 0.5 ml of 100mM KPO_4 (pH7.0), 1mM DTT. The protoplasts are lysed by performing three freeze-thaw cycles of 3 minutes at -80^{o}C followed by 3 minutes in a room temperature water bath. The tubes are centrifuged at 4 ^{o}C to remove the cell debris and luciferase assays are performed on the supernatants using the Luciferase Assay System (Promega, Madison, WI). Luminescence is measured for 10 seconds on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The results of the transient experiments are shown in the following Table.

Plasmid	Luciferase light units	times control
pAT162	545078	
PAT163 (control)	2913	
pAT152 + pAT163	212971	73x
pAT206 (control)	1372	
pAT152 + pAT206	23345	18x
pAT207 (control)	1381	
pAT152 + pAT207	52217	38x
pAT152 + pAT210	5847	
pAT152 + pAT211	6302	
pAT152 + pAT213	3147	

Example 4: **Construction of a Recipient Vector Containing Wild-type and Modified *FRT* sites**

A complete 48 bp wild-type *FRT* site is assembled by annealing and kinasing two oligonucleotides (5' - CGA AGT TCC TAT TCC GAA GTT CCT ATT CTC TAG AAA GTA TAG GAA CTT CG - 3', SEQ ID NO:17) and (5' - AAT TCG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCG GTA C - 3', SEQ ID NO:18). In order to place the *FRT* site at the 3' end of a 35S terminator, the double-stranded oligonucleotide with 5' *KpnI* and 3' *EcoRI* sticky ends is ligated into the *KpnI-EcoRI* sites of pCIB710 (Rothstein et al. (1987) Gene 53: 153-161). A *UBQ3* promoter luciferase gene fusion with the *FRT* site at the 3' end of the 35S terminator is constructed by first subcloning the terminator and FRT site as a *BamHI-EcoRI* band into the respective sites of pbluescript sk+ (Stratagene, LaJolla, CA), excising with *NotI-EcoRI*, then ligating this fragment along with the *UBQ3* promoter and luciferase gene from pPH121 as a *HindIII-NotI* band into the *HindIII-EcoRI* sites of pbluescript sk+. The *UBQ3* promoter luciferase fusion with a wild-type *FRT* site at the 3' end of a 35S terminator is inserted as a *EcoRI* fragment into pCIB730 (Rothstein et al., 1987), a binary vector lacking a *nptII* gene, yielding pAT135. A 48 bp spacer mutant *FRT* site (F^5) is assembled by annealing and kinasing two oligonucleotides (5'-TCG ACG AAG TTC CTA TTC CGA AGT TCC TAT TCT TCA AAA GGT ATA GGA ACT TCG-3', SEQ ID NO:19) and (5'-AAT TCG AAG TTC CTA TAC CTT TTG AAG AAT AGG AAC TTC GGA ATA GGA ACT TCG-3', SEQ ID NO:20). The mutant *FRT* - F^5 site is cloned at the 3' end of a *nos* promoter, *nptII* gene, *nos* terminator gene cassette by ligating the double-stranded oligonucleotide with 5' *SalI* and 3' *EcoRI* sticky ends into the respective sites of a plasmid containing the entire nptII gene cassette from pCIB10 (Rothstein et al., 1987) excised as a *SacII-EcoRI* band and cloned into the respective sites of pbluescript sk+. The *nos* promoter *nptII* gene cassette flanked on the 3' end with the mutant *FRT* - F^5 site is excised with *SacI-SalI* from this vector and ligated into the respective sites of pAT135. This final vector contains the *nptII* gene and luciferase gene cassettes in divergent orientation flanked by a wild-type and a mutant *FRT* - F^5 site at either end and is known as pAT136.

Example 5: **Plant Transformation and Selection of pAT136 Transformants**

Agrobacterium tumefaciens strain A136 containing the helper plasmid pCIB542 is transformed with pAT136. Leaf disc transformation of *Nicotiana tabacum* c.v. Petit Havana (SR1) (Maliga et al. (1973) Nature 244: 29-30) is performed essentially as described by Horsch et al. (1988) Plant Molecular Biology Manual A5: 1-9. Kanamycin resistant tobacco transformants are screened for luciferase activity using a luciferase assay system kit (Promega, Madison, WI). Genomic Southern analysis of luciferase expressing transformants is performed to determine copy number and whether the left border of the T-DNA is intact. Genomic DNA is digested with *BamHI* and Southern blots are probed with a *nptII* probe to determine copy number. The presence of a 1.45 kb band in *HindIII-BamHI* digests probed with *nptII* confirmed the presence of the left border. Single-copy, luciferase expressing pAT136 tobacco transformants with intact left borders are identified and maintained in vitro. Clonal plants are sent to the greenhouse and crossed to wild-type SR1 plants to maintain the hemizygous transgene.

Example 6: **Construction of a Donor Vector Containing Wild-type and Mutant *FRT* Sites**

A *nos* promoter-*bar* gene cassette flanked by a mutant *FRT* site (F^3) on the 5' end and a wild-type *FRT* site on the 3' end is constructed by first subcloning the *nos* promoter and *bar* gene from pGPTV-BAR (Becker et al. (1992) PMB 20: 1195-1197) as a *HindIII-BamHI* fragment and the 35S terminator and wild-type FRT site as a *BamHI-EcoRI* band (used in the construction of pAT136) into *HindIII-EcoRI* cut pbluescript SK+. A 48 bp mutant F^3 site is assembled by annealing and kinasing the two oligonucleotides (5' - TCG ACG AAG TTC CTA TTC CGA AGT TCC TAT TCT TCA AAT AGT ATA GGA ACT TCA - 3', SEQ ID NO:21) and (5'-AGC TTG AAG TTC CTA TAC TAT TTG AAG AAT AGG AAC TTC GGA ATA GGA ACT TCG - 3', SEQ ID NO:22). The mutant F^3 site is added to the 5' end of the *nos* promoter by ligation of this double-stranded oligonucleotide containing 5' *SalI* and 3' *HindIII* sticky ends into the respective sites of the previous vector. The *SalI* site at the 5' end of the F^3 site is adaptored to *SpeI* and the entire bar gene cassette flanked by the *FRT* sites is excised as a *SpeI* fragment and inserted into the *XbaI* site of a vector containing a *UBQ3* promoter GUS gene with a F^5 mutant site at the 3' end of the *nos* terminator (the *UBQ3* promoter GUS gene and *nos* terminator from ppeh27 (GUS) as a

XbaI-EcoRI band and the F^5 site as an *EcoRI-SalI* fragment into *XbaI-SalI* cut pbluescript sk+). This final vector containing the bar gene and GUS genes in divergent orientation separated by a mutant F^3 site and flanked by a wild-type and a mutant F^5 site on either end is known as pAT158.

Example 7: **Protoplast Transformation**

Protoplasts from young leaves of pAT136 transformants are transformed by the polyethylene glycol (PEG)-mediated DNA transformation method as described by Shillito and Saul. Agarose-embedded protoplasts are put in a 1:1 mixture of K3A medium and H460 medium with 5 mg/l or 10 mg/l BASTA after 7 days, with weekly media changes as described. After 3 weeks on selection, individual colonies of at least 2mm are placed on solid LS medium containing 5 or 10mg/l BASTA.

Example 8: **FLP Recombinase-mediated Integration of a Transgene into a Pre-introduced Target**

Equal numbers of protoplasts from a pAT136 tobacco transformant are cotransformed with either pAT152 / pAT158 (site-directed recombination using FLP recombinase) or with pAT153 / pAT158 (control for random integrations using a non-functional recombinase). Recombinase-mediated integrations are scored as GUS positive/ luciferase negative events whereas an event from a random integration express both the GUS and luciferase genes. Confirmation of recombinase-mediated integration events are verified by PCR using a primer from the region between the wild-type *FRT* site and the right border and a primer from the GUS gene. Southern analysis is carried out to confirm such an event.

Example 9: **Construction of Positive Control and Reverse Positive Control Plasmids for Transient Expression Experiments**

A 48 bp spacer mutant *FRT* site (F^5) (Schlake & Bode, Biochemistry 33, 12746-12751 (1994)) is cloned as an oligonucleotide into the *ClaI* site of the intron within the leader of the *Arabidopsis* ubiquitin-3 gene promoter/leader plasmid pPEH28, a derivative of pSPORT1 (BRL, Bethesda, MD) also comprising a *nos* terminator. The F^5 oligonucleotide top and bottom strands are shown below (mutant core region shown in bold; lower case nucleotides

represent the single-stranded portion of the oligo that forms a ClaI cohesive end; additional nucleotides which form an *EcoRI* site are italicized):
5'-cga t*GA ATT C*GA AGT TCC TAT TCC GAA GTT CCT ATT C**TT CAA AG**G GTA TAG GAA CTT C-3' (top strand, SEQ ID NO:23) and 5'-CGG AAG TTC CTA TAC CTT TTG AAG AAT AGG AAC TTC GGA ATA GGA ACT TC-3' (bottom strand, SEQ ID NO:24).

After ligation of the F^5 oligo pair into the *ClaI* site of pPEH28, a clone named UbqF5nos is identified with the F^5 site oriented toward the ubiquitin-3 promoter by mapping its asymmetric *EcoRI* site and its structure was confirmed by DNA sequencing. Next, a 1745 bp *BamHI* fragment of pAT134 containing a 35S leader sequence and the coding sequence of the firefly luciferase gene is ligated into the *BamHI* site of UbqF5nos. A clone with the luciferase coding region in correct orientation with respect to the UBQ3 promoter is identified by mapping and named UBQ3F5Lucnos.

In order to place a wild-type *FRT* site (F^0) 5' to the *UBQ3* promoter, an oligo pair containing the 48 bp sequence of the wild type FRT site, F^0, flanked by *XhoI* cohesive ends is inserted into the *XhoI* site of UbqF5Lucnos. The oligonucleotide top and bottom strand sequences are shown below with the cohesive *XhoI* end in lower case, an *AatII* cut site, a (C) at the 5' end and a (G) at the 3' end to destroy the *XhoI* site, and the F^0 site in standard type with its wild type core region in bold type:
5'-tcg a(C)*G ACG TC*G AAG TTC CTA TTC CGA AGT TCC TAT TC**T CTA GAA** **AG**T ATA GGA ACT TC(G)-3' (top strand, SEQ ID NO:25) and 5'-TCG ACG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCG ACG TCG-3' (bottom strand, SEQ ID NO:26).

The orientation of F^0 is determined by mapping the asymmetric *AatII* site and confirmed by DNA sequencing. The two plasmids formed are called F0fwdUBQ3F5Luc (with the F^0 and F^5 sites in parallel orientation) and F0revUBQ3F5Luc (F^0 and F^5 sites in opposite orientation). Removal of the promoter from this construct by *Asp718I* digestion and replacing it with spacer DNA forms a plasmid with substantially reduced but still significant luciferase transient expression in plant cells. One possible cause of this minimal promoter activity is a TATA-like sequence within the terminal region of the UBQ3-intron. The high AT region at the 3' end of the UBQ3 intron is removed by digestion with *ClaI* and *NcoI*, and replaced by an oligonucleotide pair constructed to fit into the same sites (top strand and bottom strand are shown below, cohesive end in lower case) to form a truncated intron

structure that we designated as dInt: 5'-cat gGA CCT GAA ACA AAA CAA T-3' (top strand, SEQ ID NO:27) and 5'-CGA TTG TTT TGT TTC AGG TC-3' (bottom strand, SEQ ID NO:28).

The resulting modified plasmids are named the Positive Control (F0UBQ3F5dIntLUC) and Reverse Positive Control (F0revUBQ3F5dIntLuc).

Example 10: **Construction of Recipient and Reverse Recipient Vectors**

The *UBQ3* promoter is deleted from the Positive Control and Reverse Positive Contol plasmids by digestion with *Asp 718I*, and a self-complementary oligonucleotide (5'-gta cAG ATC T-3', SEQ ID NO:29) is ligated into their cohesive ends, converting their *Asp718I* sites to *BglII* sites. In the resulting plasmids, F0BgF5dIntLUC and F0revBgF5dIntLuc, the *BglII* site is opened and a 518bp *BglIII* fragment of the *bar* gene from pAT 157 is ligated between the F^0 and F^5 sites, introducing "spacer" DNA and forming the Recipient (F0SpF5dIntLuc) and Reverse Recipient (F0RevSpF5dIntLuc) vectors.

Example 11: **Construction of Donor and Reverse Donor Vectors**

From the Positive Control and Reverse Positive Control vectors, *EcoRI* is employed to remove the nos terminator, luciferase coding region, 35S leader and the 3' end of the truncated intron, sparing the F^5 site. This forms the Donor (F0UBQ3F5) and Reverse Donor (F0revUBQ3F5) vectors.

Example 12: **FLP Mediated Cassette Exchange between Recipient and Donor Vectors of Examples 10 and 11**

For Biolistic delivery of DNA, BY2 tobacco suspension cells are spread onto filters on culture medium containing 12% sucrose (which acts as an osmoticum prior to bombardment). Plasmid DNA of the Reverse Recipient, Reverse Donor and Reverse Positive Control vectors is precipitated onto <1.0µm gold beads (Crescent Chemical Company, Hauppauge, NY) as described by Goff, S. et. al. (*EMBO J* 9, 2517-2522, 1990) The DNA concentration used for the Reverse Recipient, Reverse Donor and Reverse Positive Control vectors is 0.1mg /6 shots and the DNA concentration used for the FLP expression vector (pAT152) is 0.5 mg/6 shots. Each filter with BY2 cells is bombarded one

time using 650-1,100 psi rupture discs. Three replicates are performed for each treatment with one filter being one replicate. Tissue is assayed for transient luciferase expression at ~16 hours post-bombardment. Cell lysates are obtained using the Luciferase Assay System (Promega, Madison, WI). Luminescence is measured for 10 seconds on a Monolight 2010 luminometer as described in Example 3. Cell lysates are measured for soluble protein using the Bio-Rad Protein Assay System (Bio-Rad, Hercules, CA) and luciferase specific activity is reported as light units (LU) per ug soluble protein.

The results of the transient experiment are presented in below:

Vector	Luciferase Specific Activity
Reverse Recipient + Reverse Donor	152 ± 33 LU/ug protein
Reverse Recipient + Reverse Donor + pAT 152	467 ± 105 LU/ug protein
Positive Control	48,734 ± 9,658 LU/ug protein

Example 13: **Construction of an *NptII* Expression Vector with a Modified *FRT* Site in the Intron of the Gene Coding Region**

The *nos* promoter and *nptII* gene are excised from pGPTV–Kan (Becker, D. et al, Plant Molecular Biology 20: 1195-1197,1992) as a 1403 bp *BamHI– ClaI* fragment and ligated into the *BamHI – ClaI* sites 5' of the Act2 terminator (An et al. (1996) Plant J. 10: 107-121) forming an NPTII expression cassette, NosNPTact. For insertion of an intron, part of the *nos* promoter and the entire *npt* gene coding sequence is cloned as a 918 bp *NheI – BamHI* fragment into pUC18 cloning vector (NEB, Beverly, MA) to which is first added an *NheI* site by oligonucleotide insertion. An intron from *Solanum tuberosum* (Eckes, P., Rosahl S, Schell J, Willmitzer L (1986) Mol Gen Genet 199:216-224) is PCR amplified, cloned into pCR2.1-TOPO2 vector (Topo2 TA Cloning Kit, In Vitrogen), excised precisely as a SnaBI/PvuI (blunt) fragment and cloned into the *BsaAI* site of the *npt* gene coding sequence. A clone with the correct orientation of the intron is identified by mapping and confirmed by DNA sequencing. An oligonucleotide pair comprising a modified *FRT* site (F^1, F^2, F^3, F^4or F^5, Schlake & Bode, Biochemistry 33, 12746-12751 (1994)) is introduced into the cohesive ends of the *MfeI* site of the intron of *nptII*, utilizing one of the synthetic oligonucleotides depicted below, also carrying the AATT cohesive end). A clone with the *FRT* site in the correct orientation is identified by mapping and confirmed by sequencing. For example, for the F^1 Core Sequence, the following oligonucleotides are used (*NotI* and

EcoRI sites italized, Core Sequence in bold): 5'-aat tGC *GGC CGC* GAA GTT CCT ATT CCG AAG TTC CTA TTC **TCT AGA TA**G TAT AGG AAC TTC *GAA TTC*-3' (top strand, SEQ ID NO:30) and 5'-AAT TGA ATT CGA AGT TCC TAT ACT ATC TAG AGA ATA GGA ACT TCG GAA TAG GAA CTT CGC GGC CGC-3' (bottom strand, SEQ ID NO:31).

Oligonucleotides for F^2 through F^5 are identical to F^1 except in the *FRT* Site Core Sequence shown below:

Oligonucleotide	Core Sequence
F^1	5'-TCTAGATA-3' (SEQ ID NO:6)
F^2	5'-TCTACTTA-3' (SEQ ID NO:4)
F^3	5'-TTCAAATA-3' (SEQ ID NO:3)
F^4	5'-TCTAGAAG-3' (SEQ ID NO:5)
F^5	5'-TTCAAAAG-3' (SEQ ID NO:2)

To reconstruct the original expression cassette, the *nptII* gene, now containing an intron with a modified *FRT* site, is excised as a 1171 bp *NheI – BamHI* fragment and re-ligated into the same sites of the original expression vector. From this cassette, the 3' untranslated region of Actin-2 is removed by *BamHI – SacI* digestion and replaced by a 343 bp *BglII – SacI* fragment containing the PAL 3' untranslated region (Wanner, L.A. et. al., Plant Mol. Biol. 27 (2), 327-338 (1995). This produces a family of clones whose members are named NosNptIntFxPal, where x is 1, 2, 3, 4 or 5 as defined above.

For delivery into plant cells using *Agrobacterium tumefaciens,* the desired NosNptIntFxPal is cloned into a binary vector, such a binary vector well-known in the art, as a 2073 bp *SacI – XhoI* fragment and the resulting plasmid is transformed into a helper *Agrobacterium* strain suitable for the plant host to be transformed, for example LBA4404 or A136(pCIB542) or MP90.

Example 14: **Construction of a FLP Expression Cassette**

The FLP gene coding sequence is cloned from pAT152, described in Example 1, into pUC18 as a 1395 bp *Asp718I - BamHI* fragment. The *S. tuberosum* intron described in Example 13 is introduced into the *EcoRV* site of the FLP coding sequence and a clone with the correct orientation is identified by mapping and confirmed by sequencing. The *Asp718I*

site is converted into a *BamHI* site and the FLP coding sequence, now containing an intron, is cloned as a 1589 bp *BamHI* fragment into the *BamHI* site between the *Arabidopsis* Actin-2 promoter and terminator (An et al. (1996) Plant J. 10: 107-121). A clone with the correct orientation of FLP in the expression cassette is identified by mapping and designated ActIntFLP.

For delivery into plant cells using *Agrobacterium tumefaciens*, a 3665 bp *SacI – XhoI* fragment of ActIntFLP is cloned into a binary vector, and the resulting plasmid is transformed into a helper *Agrobacterium* strain suitable for the plant host to be transformed, for example LBA4404 or MP90.

Example 15: **Construction of an Excisable FLP Expression Cassette**

Integration of the FLP expression cassette into the plant genome is not needed and indeed not desirable during the process of site-specific integration. A way to reverse any such integration is to flank the cassette by parallel matching FRT sites. Integration of such a construct is reversed by FLP recombinase, which efficiently catalyzes excision of DNA between parallel matching FRT sites.

Plasmid ActIntFLP (Example 14) is digested by *Asp718I* and *ApaI* and a family of oligonucleotide pairs are individually ligated into these sites forming a *FRT* site that corresponds to F^0 or F^1- F^5. The oligonucleotide pair is indicated below. (Top strand of F is shown; bottom strand is complementary except for the cohesive ends shown in lower case typescript at each end of the top strand. The sequence of eight N's in the center represents any desired core sequence, TCTAGAAA for F^0 or the various F^1- F^5 core sequences in example 13. The added [C] residue near the 5'-end is included to spare the *Asp718I* site for future use, the *Asp7181* and *ApaI* cohesive ends are shown in lower case: 5'-gta c[C]G AAG TTC CTA TTC CGA AGT TCC TAT TCN NNN NNN NGT ATA GGA ACT TCg gcc-3' (top strand, SEQ ID NO:32) and 5'-G AAG TTC CTA TAC NNN NNN NNG AAT AGG AAC TTC GGA ATA GGA ACT TCG-3' (bottom strand, SEQ ID NO:33).

The resulting product structure is identified by survival of an Asp718I site and loss of the *ApaI* site, and confirmed by sequencing. The family of intermediates is designated FxActIntFlp, and is next digested with *SacI* and *NotI* to introduce the second (matching) F^x site as an oligonucleotide pair shown below. Both strands of the oligonucleotides are shown for clarity: 5'-GAA GTT CCT ATT CCG AAG TTC CTA TTC NNN NNN NNG TAT

AGG AAC TTC [GC]-3' (top strand, SEQ ID NO:34) and 5'-tcg aCT TCA AGG ATA AGG CTT CAA GGA TAA GNN NNN NNN CAT ATC CTT GAA G[CG] ccg g-3' (bottom strand, SEQ ID NO:35). The Ns represents a *FRT* core sequence that matches the F^x core sequence at the other end of the same clone. Flanking sequence in lower case represents the cohesive ends for *SacI* (3'end of bottom strand) and *NotI* (5'end of bottom strand). The [GC] sequence at the right end is added in order to preserve the *NotI* site for future use. A clone with the correct oligo insert is identified by loss of the *SacI* site and confirmed by DNA sequencing. The resulting excisable donor family is termed: FxActIntFlpFx.

For delivery into plant cells using *Agrobacterium tumefaciens*, an *Asp718I-NotI* fragment of FxActIntFLPFx is cloned into a binary vector between Bsp120I and SacI sites and the resulting plasmid is transformed into a helper *Agrobacterium* strain suitable for the plant host to be transformed, for example LBA4404 or MP90.

Example 16: **Construction of a Family of Recipient Vectors Containing a *bar* Gene Expression Cassette, a Partial *nptII* Gene Expression Cassette and Wild-type and Modified *FRT* Sites**

A family of recipient vectors is constructed with a *bar* gene expression cassette flanked by a wild-type and a modified *FRT* site with the sites either in parallel or opposite orientation relative to each other. Outside of the region flanked by the two *FRT* sites is the DNA sequence in the *nptII* expression vector described in Example 13 which is 3' of the modified *FRT* site.

The family of recipient vectors is constructed starting from pNOV111 containing the UBQ3IntBARnos expression cassette. The *RsrII and XhoI* sites of pNOV 111 are changed by oligo converters into *HindIII and BamHI* sites respectively. The cassette is then excised as a 2605 bp *HindIII – BamHI* fragment into pBluescriptSK (Stratagene, Beverly, MA), forming pBS.UBQ3IntBARnos. The wild-type *FRT* site, F^0, is ligated into the *XhoI* site of pBS.UBQ3IntBARnos as an oligo pair with flanking XhoI cohesive ends (top strand: 5'-tcg aGA AGT TCC TAT TCC GAA GTT CCT ATT CTC TAG AAA GTA TAG GAA CTT CAC *GCG T*-3', SEQ ID NO:36, core sequence in bold and *MluI* site italized, and bottom strand: 5'-TCG AAC GCG TGA AGT TCC TAT ACT TTC TAG AGA ATA GGA ACT TCG GAA TAG GAA CTT C-3', SEQ ID NO:37) and clones with F^0 in either orientation are identified by mapping the asymmetric *MluI* site. The products are designated

UBQ3IntBARnosF0fwd for the orientation of the top strand opposite that of the *bar* gene and UBQ3IntBARnosF0rev for the orientation parallel to *bar*.

The terminal half of the *nptII* gene cassette containing the modified *FRT* site is excised from NosNptIntFxPal (described in Example 13) as a 840 bp *NotI – SacI* fragment and is ligated into the *NotI – SacI* sites of UBQ3IntBARnosF0fwd or UBQ3IntBARnosF0rev to form two Recipient Vector families, F0fwdUBQ3IntBARnosFx[3'Int][3'Npt]Pal and F0revUBQ3IntBARnosFx[3'Int][3'Npt]Pal. (The brackets enclosing 3'Int and 3'Npt are to indicate that only the 3'-terminal part of each of these structures is present in the plasmid. A promoter, the missing part of the intron and the missing portion of Npt will be restored by site-specific integration where the recombinase is FLP. F^0 and F^x sites are oriented parallel to one another in F^0fwd Recipient and oppositely to one another in F^0rev Recipient.) In different family members, the modified *FRT* site F^x is either F^1, F^2, F^3, F^4or F^5.

For delivery into plant cells using *Agrobacterium*, the 3546 bp *SacI-MluI* fragment (F^0rev Recipient) or *SacI-XhoI* fragment (F^0fwd Recipient) is cloned into a binary vector at its *SacI- MluI* sites or *SacI-SalI* sites respectively. The resulting plasmids are transformed into a helper *Agrobacterium* strain suitable for the plant host to be transformed, for example LBA4404 or A136(pCIB542) or MP90.

Example 17: **Construction of a Family of Recipient Vectors Containing a *bar* Gene Expression Cassette, a FLP Expression Cassette, a Partial *nptII* Gene Expression Cassette and Wild-type and Modified *FRT* Sites**

A family of recipient vectors is constructed that is identical to those of Example 16, except inserted between F^x and the UBQ3 promoter of the *bar* gene expression is a FLP gene expression cassette with an intron in the FLP coding sequence oriented in the same direction as *bar*.

This family of recipient vectors is constructed by ligating the FLP gene expression cassette of Example 14 as a 3581 bp *XbaI* fragment into the *XbaI* site of pBS.UBQ3IntBARnos described in Example 16. An intermediate is identified with the *bar* and FLP expression cassettes both oriented in the same direction and is called ActFLPIntTactUBQ3IntBARnos. The F^0 site and the terminal half of the *nptII* gene cassette containing the modified *FRT* site are inserted into ActFLPIntTactUBQ3IntBARnos in separate steps exactly as described above in Example 16, forming the family of recipient vectors F0fwdUBQ3IntBARnosActFLPIntTactFx[3'Int][3'Npt]Pal and

F0revUBQ3IntBARnosActFLPIntTactFx[3'Int][3'Npt]Pal. The modified *FRT* site F^x is either F^1, F^2, F^3, F^4or F^5.

For delivery into plant cells using *Agrobacterium,* the 7127 bp *SacI-MluI* fragment of the FLP-containing F^0rev Recipient Vectors and the corresponding 7127 bp *SacI-XhoI* fragment of the F^0fwd Recipient Vectors are cloned into a binary vector at its *SacI- MluI* sites and *SacI-SalI* sites respectively. The resulting plasmids are transformed into a helper *Agrobacterium* strain suitable for the plant host to be transformed, for example LBA4404 or A136(pCIB542) or MP90.

Example 18: **Construction of a Family of Donor Vectors for site-specific integration with the Recipient Families of Examples 16 and 17**

A family of donor vectors is constructed for restoration of *nptII* gene expression by site-specific integration with a recipient vector which has corresponding *FRT* sites.

The oligonucleotide pair comprising the wild-type *FRT* site, F^0, described in Example 16 above, is ligated into the *XhoI* site of pBluescript forming pBS.F0fwd and pBS.F0rev. The orientation of the F^0 site is determined by mapping using its asymmetric *MluI* site and confirmed by sequencing. The F^0fwd construct has the F^0 core arrow (Example 16) in the direction of *KpnI* to *SacI* of the polylinker, and the F^0rev construct has the opposite orientation. The 1272 bp *ClaI-EcoRI* fragment of NosNptIntFxPal (Example 13) containing the *nos* promoter, *nptII* gene coding sequence, and part of the intron up to and including the modified *FRT* site is ligated into the corresponding sites of pBS.F0fwd and pBS.F0rev, forming two donor vector families, F0fwdNos[5'Npt][5'Int]Fx and F0revNos[5'Npt][5'Int]Fx. (The brackets enclosing 5'Int and 5'Npt are to indicate that only the 5'-proximal part of each of these structures is present in the plasmid. These are precisely the components necessary to complete the corresponding 3'-terminal parts in the recipient vectors of Example 16, in order to form an intact *nptII* coding sequence.) The modified *FRT* site site F^x is either F^1, F^2, F^3, F^4or F^5. As for the Recipient families, the Donor family with F^0fwd has F^0 and F^x sites in parallel orientation while the family with F^0rev has F^0 and F^x sites oppositely oriented.

Example 19: **Construction of an Excisable Donor Vector for site-specific integration**

When donor DNA is delivered by *Agrobacterium*, initially it is in single-stranded linear form, and therefore presumably not a proper substrate for site-specific integration. In order to facilitate production of a double-stranded circular form of donor DNA, a second F^0 site is introduced into the donor construct that allows FLP-mediated circularization of the donor construct between parallel F^0 sites.

A wild-type F^0 site is ligated as an oligonucleotide pair into the *XmaI* site adjacent to the modified *FRT* site in the two families of donors described in Example 18. The F^0 oligonucleotide is shown below (top strand: 5'-ccg gGA AGT TCC TAT TCC GAA GTT CCT ATT C**TC TAG AAA** GTA TAG GAA CTT C*GA GCT C*(T)-3', SEQ ID NO:38, F^0 Core Sequence in bold, and the bottom strand: 5'-CCG GAG AGC TCG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TC-3', SEQ ID NO:39, which also bears an *XmaI* cohesive end but is otherwise complementary; the extra (T) residue outside the *SacI* site in the oligo is added in order to inactivate the *XmaI* site on one end of the insert.

After insertion of the oligo pair into the F^0fwd donor family, clones with the two F^0 sites in parallel orientation are identified by mapping the asymmetric *SacI* site and confirmed by sequencing. This forms the new donor family F0fwdNos[5'Npt][5'Int]FxF0fwd. Similarly, from the F^0rev donor family a new F0revNos[5'Npt][5'Int]FxF0rev donor family is identified.

For delivery into plant cells using *Agrobacterium*, the F^0fwd F^0 donor family is subcloned as a *SacI-Asp718I* fragment into the corresponding sites in a binary vector. The F^0rev F^0 donor family is subcloned as an *XmaI-Asp718I* fragment into the corresponding sites in a binary vector. The resulting plasmids are transformed into a helper *Agrobacterium* strain suitable for the plant host to be transformed, for example LBA4404 or A136(pCIB542) or MP90.

Example 20: **Plant Transformation and Selection of BASTA Resistant F^0fwd and F^0rev Recipient Family Transformants**

Agrobacterium tumefaciens strain MP90 is transformed with the binary vector containing F0fwdUBQ3BARnosFx[3'Int][3'Npt]PaI or F0revUBQ3BARnosFx[3'Int][3'Npt]PaI (Example 16). Transformation of *Nicotiana tabacum* suspension cell line, BY2, with the above

Agrobacterium strains is performed essentially as described by An, G. et al (Plant Molecular Biology Manual A3: 1-19, Gelvin, S.B. and Schilperoort, R.A. Eds, Kluwer Academia Publishers, Dordrecht/Boston/London, 1987). BASTA resistant tobacco transformants are selected and subcultured on MS media supplemented with 0.2 mg/l 2,4-D, 5-10 mg/l BASTA and 500mg/l carbenicillin.

Example 21: **Plant Transformation and Selection of BASTA Resistant F^0fwd and F^0rev Recipient Family Transformants Containing a FLP Gene Expression Cassette**

Agrobacterium tumefaciens strain MP90 is transformed with a binary vector containing F0fwdUBQ3BARnosActFLPTactFx[3'Int][3'Npt]Pal or F0revUBQ3BARnosActFLPTactFx[3'Int][3'Npt]Pal (Example 17). Transformation of *Nicotiana tabacum* suspension cell line, BY2, with the above *Agrobacterium* strains is performed essentially as described by An, G. et al (Plant Molecular Biology Manual A3: 1-19, Gelvin, S.B. and Schilperoort, R.A. Eds, Kluwer Academia Publishers, Dordrecht/Boston/London, 1987). BASTA resistant tobacco transformants are selected and subcultured on MS media supplemented with 0.2 mg/l 2,4-D, 5-10 mg/l BASTA and 500mg/l carbenicillin. Transient assays are conducted to measure the relative level of FLP gene expression in recipient vector transformants as described in Example 12. Plasmid DNA of pAT163, described in Example 2, is used at a concentration of 1.0µg per 3 shots and bombarded with one shot into one replicate of each recipient vector transformant. Luciferase specific activity is determined as described in Example 12 and represents the relative level of FLP recombinase activity.

Example 22: **FLP Mediated Cassette Exchange between Donor Vectors and BASTA Resistant Recipient Transformants**

Tobacco transformants containing recipient vector DNA of Example 20 undergo site-specific integration with donor vector DNA (Examples 18 and 19) in the presence of FLP recombinase forming kanamycin resistant transformants. The FLP gene is introduced into the recipient transformant tobacco cells ($BASTA^R$, Kan^S) as plasmid DNA of ActIntFLP or FxActIntFLPFx (Examples 14 and 15) via microprojectile particle bombardment. Plasmid DNA of donor vectors F0fwdNos[5'Int][5'Npt]Fx, F0revNos[5'Int][5'Npt]Fx, F0fwdNos[5'Int][5'Npt]FxF0fwd, or F0revNos[5'Int][5'Npt]FxF0rev is co-precipitated onto

gold beads with ActIntFLP or FxActIntFLPFx and co-bombarded into the transformed cells using the DNA precipitation and bombardment procedures described in Example 12. The core sequence of the F^x sites and the orientation of the F^0 and F^x sites are identical in the recipient transformant and donor DNA to allow for site-specific integration. In the presence of FLP recombinase, the DNA flanked by F^0 and F^x in the donor vector undergoes FLP mediated cassette exchange with the DNA flanked by the same sites in the recipient transformants, giving rise to kanamycin resistant site-specific integration transformants selected on MS medium supplemented with 0.2mg/l 2,4-D, 100mg/l kanamycin and 500mg/l carbenicillin. PCR analysis of the kanamycin resistant site-specific integration transformants further substantiates that the donor DNA is now integrated between the F^0 and F^x sites of a previously integrated recipient vector, forming an intact *nptII* gene expression cassette as described in Example 18. If the recipient transformant contains only one copy of the recipient vector DNA, then the *bar* gene cassette is excised during site-specific integration with the donor vector, forming $BASTA^S$ tobacco cell lines. The recipient transformants are therefore, thoroughly washed prior to bombardment to eliminate any residual BASTA.

In a separate transformation experiment, the FLP gene is introduced into the recipient transformant tobacco cells ($BASTA^R$, Kan^S) as T-DNA from *A. tumefaciens* strain MP90, transformed with a binary vector containing ActIntFLP or FxActIntFLPFx (Examples 14 and 15). The donor DNA (Example 19) is introduced into the tobacco cell simultaneously as a T-DNA from a different *A. tumefaciens* strain MP90, transformed with a binary vector containing F0fwdNos[5'Int][5'Npt]FxF0fwd or F0revNos[5'Int][5'Npt]FxF0rev. The recipient transformants are thoroughly washed prior to co-cultivation with the above *A. tumefaciens* strains to eliminate any residual BASTA and carbenicillin. The core sequence of the F^x sites and the orientation of all F^0 and F^x sites are identical in the recipient transformant and double-stranded circular form of donor DNA (described in Example 19) to allow for site-specific integration. Kanamycin resistant site-specific integration transformants are selected and PCR analysis is performed as described in the paragraph above.

Example 23: **FLP Mediated Cassette Exchange between Donor Vectors and BASTA Resistant Recipient Transformants Expressing FLP Recombinase**

Tobacco transformants containing recipient vector DNA of Example 21 and expressing FLP recombinase undergo site-specific integration with donor vector DNA (Examples 18 and 19) forming kanamycin resistant transformants. The donor vector is introduced into the recipient transformant tobacco cells (FLP+,BASTA^R, Kan^S) as plasmid DNA of F0fwdNos[5'Int][5'Npt]Fx, F0revNos[5'Int][5'Npt]Fx, F0fwdNos[5'Int][5'Npt]FxF0fwd, or F0revNos[5'Int][5'Npt]FxF0rev via microprojectile particle bombardment following procedures described in Example 12. The core sequence of the F^x sites and the orientation of the F^0 and F^x sites are identical in the recipient transformant and donor DNA to allow for site-specific integration. In the presence of FLP recombinase, the DNA flanked by F^0 and F^x in the donor vector undergoes FLP mediated cassette exchange with the DNA flanked by the same sites in the recipient transformants, giving rise to kanamycin resistant site-specific integration transformants selected on MS medium supplemented with 0.2mg/l 2,4-D, 100mg/l kanamycin and 500mg/l carbenicillin. PCR analysis of the kanamycin resistant site-specific integration transformants further substantiates that the donor DNA is now integrated between the F^0 and F^x sites of a previously integrated recipient vector, forming an intact *nptII* gene expression cassette as described in Example 18. If the recipient transformant contains only one copy of the recipient vector DNA, then the *bar* and FLP gene cassettes are excised during site-specific integration with the donor vector, forming BASTA^S and FLP(-) tobacco cell lines. The recipient transformants are therefore thoroughly washed prior to bombardment to eliminate any residual BASTA.

In a separate transformation experiment, the donor DNA (Example 19) is introduced into the recipient transformants expressing FLP (FLP+,BASTA^R, Kan^S) as a T-DNA from *A tumefaciens* strain MP90, transformed with a binary vector containing F0fwdNos[5'Int][5'Npt]FxF0fwd or F0revNos[5'Int][5'Npt]FxF0rev. The recipient transformants are thoroughly washed prior to co-cultivation with the above *A. tumefaciens* strains to eliminate any residual BASTA and carbenicillin. The core sequence of the F^x sites and the orientation of all F^0 and F^x sites are identical in the recipient transformant and double-stranded circular form of donor DNA (described in Example 19) to allow for site-specific integration. Kanamycin resistant site-specific integration transformants are selected and PCR analysis is performed as described in the paragraph above.

What Is Claimed is:

1. A method for site-specific modification of a plant genome comprising:
   a) obtaining a plant cell stably transformed with a first DNA sequence comprising two recombinase recognition sites which differ from one another in their spacer;
   b) introducing into said plant cell a second DNA sequence comprising the two recombinase recognition sites corresponding to the recombinase recognition sites of the first DNA sequence, in the presence of active recombinase(s);
   c) identifying a recombinase-mediated integration of the second DNA sequence at the chromosomal location of the first DNA sequence.

2. The method of claim 1, wherein said modification comprises the addition of a nucleotide sequence at the chromosomal location of the first DNA sequence.

3. The method of claim 1, wherein said modification comprises the replacement of a nucleotide sequence at the chromosomal location of the first DNA sequence by another nucleotide sequence at the same chromosomal location.

4. The method of claim 1, wherein said modification comprises the removal of a nucleotide sequence at the chromosomal location of the first DNA sequence.

5. The method of claim 1, wherein said active recombinase is transiently expressed in said plant cell.

6. The method of claim 5, wherein said active recombinase is provided to said plant cell as a messenger RNA molecule, wherein said messenger RNA molecule is translatable in said plant cell.

7. The method of claim 5, wherein said active recombinase is provided to said plant cell as active protein.

8. The method of claim 1, wherein said plant cell is stably transformed with a DNA sequence comprising the coding region of at least one recombinase, wherein said coding region is expressible in said plant cell.

9. The method of claim 1, wherein the two recombinase recognition sites are derived from two different site-specific recombination systems.

10. The method of claim 1, wherein the two recombinase recognition sites are derived from the same site-specific recombination system.

11. The method of claim 10, wherein the site-specific recombination system is selected from a group consisting of R/*RS*, Gin/*gix* and FLP/*FRT*.

12. The method of claim 11, wherein the site-specific recombination system is FLP/*FRT*.

13. The method of claim 12, wherein the spacers are selected from a group consisting of SEQ ID NO:1-6.

14. The method of claim 1, wherein the first DNA sequence further comprises outside of the DNA stretch comprised between the two recombinase recognition sites the coding region of a selectable maker gene or a portion thereof, wherein the 5' end of said coding region is adjacent to one of the recombinase recognition sites, wherein said selectable marker gene is potentially expressible in said plant cell but not expressed.

15. The method of claim 14, wherein the 3' end of said coding region is operably linked to termination signals.

16. The method of claim 14, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell.

17. The method of claim 14, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell.

18. The method of claim 14, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a negative selectable marker gene expressible in said plant cell.

19. The method of claim 14, wherein the second DNA sequence further comprises between the two recombinase recognition sites a promoter capable of directing the expression of a gene in a plant cell, wherein said promoter is adjacent to one of the recognition sites and is oriented such that it is capable of directing transcription towards the outside of the DNA stretch comprised between the two recombinase recognition sites, wherein a functional fusion between said coding region of the first DNA sequence and said promoter is created upon recombinase-mediated integration of the second DNA sequence at the chromosomal location of the first DNA sequence.

20. The method of claim 14, wherein the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell.

21. The method of claim 14, wherein the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell.

22. The method of claim 14, wherein the second DNA sequence further comprises between the two recombinase recognition sites one or more additional recombinase recognition sites different from the other two recombinase recognition sites in said DNA sequence and the coding sequence of a selectable marker gene, wherein the 5' end of said coding region is adjacent to one of the additional recombinase recognition sites, wherein said selectable marker gene is expressible in said plant cell.

23. The method of claim 14, wherein the 3' end of said coding region is operably linked to termination signals.

24. The method of claim 1, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a selectable marker gene expressible in said plant cell.

25. The method of claim 24, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell.

26. The method of claim 24, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a negative selectable marker gene expressible in said plant cell.

27. The method of claim 24, wherein the first DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell.

28. The method of claim 24, wherein the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a selectable marker gene expressible in said plant cell.

29. The method of claim 24, wherein the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a gene of interest expressible in said plant cell.

30. The method of claim 24, wherein the second DNA sequence further comprises between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell.

31. The method of claim 24, wherein the second DNA sequence further comprises between the two recombinase recognition sites one or more additional recombinase recognition sites different from the two other recombinase recognition sites of said DNA sequence.

32. A plant cell stably transformed with a DNA sequence comprising two different recombinase recognition sites.

33. The plant cell of claim 32, further comprising outside of the two recombinase recognition sites the coding region of a selectable maker gene, wherein the 5' end of

said coding region is adjacent to one of the recombinase recognition sites, wherein said selectable marker gene is potentially expressible in said plant cell but not expressed.

34. The plant cell of claim 33, wherein the 3' end of said coding region is operably linked to termination signals.

35. The plant cell of claim 33, further comprising between the two recombinase recognition sites an expression cassette comprising a visible marker gene expressible in said plant cell.

36. The plant cell of claim 33, further comprising between the two recombinase recognition sites an expression cassette comprising a selectable marker gene expressible in said plant cell.

37. The plant cell of claim 33, further comprising between the two recombinase recognition sites an expression cassette comprising a negative selectable marker gene expressible in said plant cell.

38. A plant regenerated from the plant cell of claim 32.

39. A plant obtained by the method of claim 1.

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<400> 37
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(57) Abstract

The present invention describes methods for site–directed integration of a transgene in a plant genome using site–specific recombination and site–specific recombinase systems. The invention discloses the use of two different recombinase recognition sites, in particular two recombinase recognition sites which differ in their spacers. The invention also relates to transgenic plants obtained by a method of the present invention and to their progeny.

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International Application No
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	LYZNIK ET AL: "FLP-mediated recombination of FRT sites in the maize genome" NUCLEIC ACIDS RESEARCH, vol. 24, no. 19, 1 January 1996 (1996-01-01), pages 3784-3789, XP002096357 ISSN: 0305-1048	32-38
Y	the whole document	1-8, 10-39
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 November 1999	26/11/1999

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
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E	WO 99 25840 A (BASZCZYNSKI CHRISTOPHER L ;GORDON KAMM WILLIAM J (US); LYZNIK LESZ) 27 May 1999 (1999-05-27) page 4, line 1 -page 19, line 27 ---	1-6, 9-12,32, 38,39
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